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Engineering Salt Tolerance in Plants

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Introduction

Agricultural productivity is severely affected by soil salinity, and the damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, which are predominantly sensitive to the presence of high concentrations of salts in the soil. Levels of salt inimical to plant growth affect large terrestrial areas of the world. It is estimated that more than a third of all of the irrigated land in the world is presently affected by salinity. This is exclusive of the regions classified as arid and desert lands (which comprise 25% of the total land of our planet). The loss of farmable land due to salinization is directly in conflict with the needs of the world population, projected to increase by 1.5 billion in the next 20 years, and the challenge of maintaining the world food supplies. Although famine in the world nowadays is originated by complex problems and not only by an insufficient production of food, there is no doubt that the gains in food production provided by the Green Revolution have reached their ceiling, while the world population continues to rise. Therefore, increasing the yield of crop plants in normal soils and in less productive lands, including salinized lands, is an absolute requirement for feeding the world.

The degradation of agricultural land and water supplies is a result of the intensive agricultural practices employed in developed and developing countries. Ideally, these practices should be changed to a more rational use of land and water resources; however, this change will not occur in the foreseeable future. For example, mixed

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Abbreviations: APX, ascorbate peroxidases; ATP-S, ATP sulphurylase; BADH, betaine aldehyde dehydrogenase; CAT, catalases; CDH, choline dehydrogenase; CMO, chloline monooxygenase; COD, choline oxidase; γ-ECS, γ-glutamyl-cysteine synthetase; GPX, glutathione peroxidases; GR, gluthatione reductase; GST, glutathione S-transferases; KIRCs, potassium ion inward rectifying channels; KORCs, potassium ion outward rectifying channels; PEAMT, phosphoethanolamine N-methyltransferase; P5CDS, P5CD synthase; P5CR, P5C reductase; ProDH, proline dehydrogenase; ROS, reactive oxygen species; SAT, serine acetyl transferase; SOD, superoxide dismutases; VIC, voltage-independent cation channels.

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cropping with perennials and trees would alleviate the accumulation of sodium and other salts in the upper soil layers. None the less, this kind of change in farming systems, and the development of new products, is likely to be a long and difficult process, since it will require the use of new land and will not address the problem of growing crops in land that is already compromised. The development and use of crops that can tolerate the high levels of salinity in the soils is a practical solution, at least for the time being. Although conventional breeding for salt tolerance has been attempted for a long time, the lack of success in generating tolerant varieties (given the low number of varieties released and their limited salt tolerance) would suggest that conventional breeding practices are not enough and that, in order to succeed, a breeding program should include the engineering of transgenic salt-tolerant crops.

Salinity imposes two stresses to plant tissue: a) a water deficit that results from the relatively high solute concentrations in the soil; and b) ion-specific stresses resulting from altered K^+/Na^+ ratios and Na^+ and Cl^- concentrations that are inimical to plants. As salinity stress is a continuing and increasingly deleterious obstacle to the growth and yield of crop plants, due to irrigation practices and increasing demands on fresh water supply, the engineering of salinity-tolerant crop plants has been a long held and intensively sought objective. Breeding efforts using salt-tolerant relatives of crop plants has had only limited success, although the mapping of QTLs in tomato (Foolad *et al.*, 2001) and rice (Koyama *et al.*, 2001), and methods such as somatic hybridization (Wei *et al.*, 2001) hold promise as a combination of conventional breeding and a molecular approach. The availability of genome sequence information and the utility of yeast as a model system for functional testing (and a source of genes) has facilitated a more rapid identification of desirable genes. However, traditional mutagenesis continues to be a fruitful source of discovery, as exemplified by the identification of two loci in tomato that appear to be important for salt tolerance (Borsani *et al.*, 2001). This review focuses on the recent experimentation with transgenic plants that has led to increased salinity tolerance, with emphasis on the areas of ion homeostasis, osmotic regulation, and anti-oxidant protection. There is an emerging body of work in the area of signalling and transcriptional control that has been reviewed recently (Hasegawa *et al.*, 2000; Zhu, 2001, 2002) and will not be dealt with here.

Ion homeostasis

Animal cells have adapted to live with relatively high extracellular salt concentrations. The ubiquitous plasma membrane Na^+/K^+-ATPase mediates the efflux of 3 Na^+ and the influx of $2K^+$, which is coupled to the hydrolysis of ATP. This electrogenic Na^+/K^+ exchange establishes a Na^+ gradient across the plasma membrane that is used by the cell for the regulation of nutrient uptake, volume, and pH. In contrast to animal cells, Na^+ is not essential for plants. Plants lack a plasma membrane Na^+/H^+ ATPase. Instead, they possess H^+-ATPase, which generates the H^+ electrochemical gradient that drives the transport of ions and nutrients. Although Na^+ is required in some plants, particularly halophytes (Glenn *et al.*, 1999), a high NaCl concentration is a limiting factor for plant growth. The alteration of ion ratios in the plant is due to the influx of sodium through pathways that function in the acquisition of potassium. The stealth of sodium entry is due to the similarity between the hydrated ionic radii of sodium and

potassium, which makes the discrimination between the two ions by transport proteins difficult (Blumwald *et al.*, 2000). This discrimination problem is also the basis for Na^+ toxicity, where key biochemical processes in the plant cell are inhibited by the competition by sodium for potassium binding sites. The sensitivity to salt of cytosolic enzymes is similar in both glycophytes (salt-sensitive plants) and halophytes (salt-tolerant plants), indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt (Glenn *et al.*, 1999). Strategies that plants could use in order to maintain a high K^+/Na^+ ratio in the cytosol include: i) diminishing the entry of Na^+ ions into the cells; ii) extrusion of Na^+ ions out of the cell; and iii) vacuolar compartmentation of Na^+ ions.

Under typical physiological conditions, plants maintain a high cytosolic K^+/Na^+ ratio. Given the negative membrane potential difference at the plasma membrane (-140 mV) (Higinbotham, 1973) (*Figure 12.1*), a rise in extracellular Na^+ concentration will establish a large electrochemical gradient favouring the passive transport of Na^+ into the cells. Three classes of low affinity K^+ channels have been identified. Inward rectifying channels (KIRC), such as AKT1 (Sentenac *et al.*, 1992), activate K^+ influx upon plasma-membrane hyperpolarization, and they display a high K^+/Na^+ selectivity ratio. A knockout mutant of *AKT1* in *Arabidopsis* (akt1-1) has displayed a similar sensitivity to salt as the wild type, suggesting that this channel does not play a role in Na^+ uptake (Spalding *et al.*, 1999). K^+ outward rectifying channels (KORCs) could play a role in mediating the influx of Na^+ into plant cells. KORC channels have shown a high selectivity for K^+ over Na^+ in barley roots (Wegner and Raschke, 1994), and a somewhat lower K^+/Na^+ selectivity ratio in *Arabidopsis* root cells (Maathuis and Sanders, 1995). These channels, which open during the depolarization of the plasma membrane (i.e. upon a shift in the electrical potential difference to more positive values), could mediate the efflux of K^+ and the influx of Na^+ ions (Maathuis and Sanders, 1997). Voltage-independent cation channels (VIC) in plant plasma membranes have been reported (de Boer and Wegner, 1997; Roberts and Tester, 1997). These channels have a relatively high Na^+/K^+ selectivity, are not gated by voltage, and provide a pathway for the entry of Na^+ into plant cells (Maathuis and Amtmann, 1999).

Sodium ions can enter the cell through a number of low- and high-affinity K^+ carriers. Among these is AtHKT1 from *Arabidopsis*, which has been shown to function as a selective Na^+ transporter, and to a lesser extent, to mediate K^+ transport (Uozumi *et al.*, 2000). Recently, AtHKT1 was identified as a regulator of Na^+ influx in plant roots. This conclusion was based on the capacity of *hkt1* mutants to suppress Na^+ accumulation and sodium hypersensitivity in a *sos3* (*s*alt-*o*verly-*s*ensitive) mutant background (Rus *et al.*, 2001), suggesting that AtHKT1 is a salt tolerance determinant that controls the entry of Na^+ into the roots.

Sodium extrusion from plant cells is powered by the operation of the plasma membrane H^+-ATPase generating an electrochemical H^+ gradient that allows plasma membrane Na^+/H^+ antiporters to couple the passive movement of H^+ inside the cells, along its electrochemical potential, to the active extrusion of Na^+ (Blumwald *et al.*, 2000). Recently, *AtSOS1* from *Arabidopsis thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiport that has a significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi *et al.*, 2000). Analysis of *SOS1* promoter-GUS transgenic *Arabidopsis* plants showed expression in epidermal

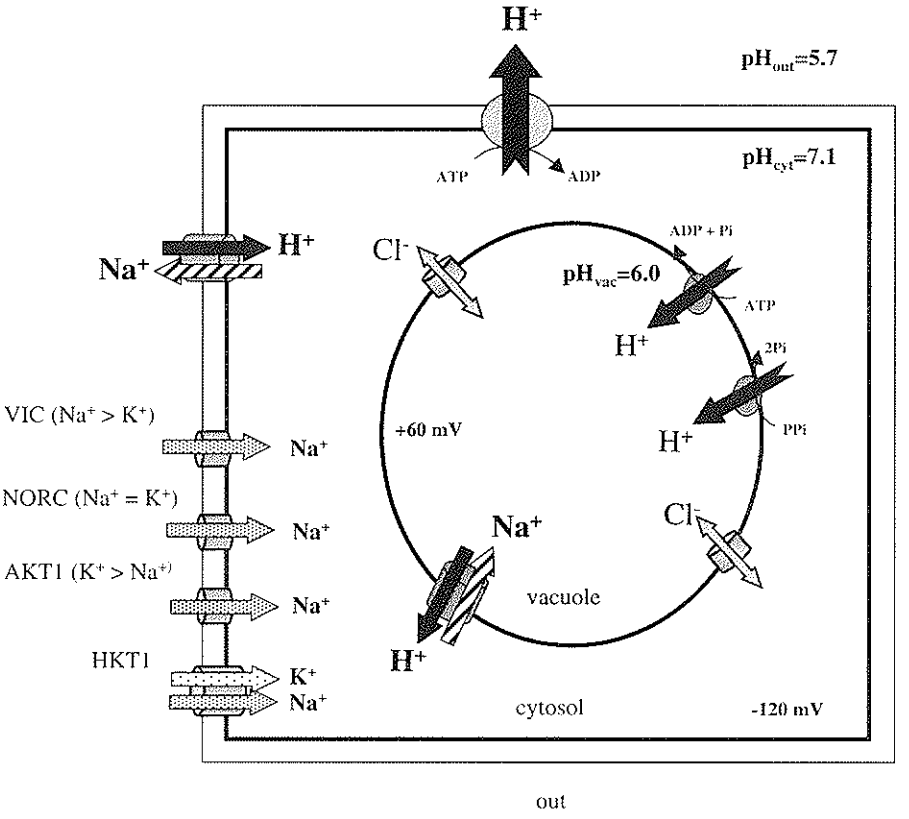

Figure 12.1. Transport systems at the plasma membrane and the tonoplast of plant cells.

cells of the root tip and in parenchyma cells at the xylem/symplast boundary of roots, stems, and leaves (Shi *et al.*, 2002).

The compartmentation of Na^+ ions into vacuoles provides an efficient mechanism to avert the toxic effects of Na^+ in the cytosol. The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PPiase (Blumwald, 1987). The overexpression of an *AtNHX1*, a vacuolar Na^+/H^+ antiporter from *Arabidopsis,* in *Arabidopsis* has resulted in transgenic plants that were able to grow in high salt concentrations (Apse *et al.*, 1999). Additional evidence supporting the role of vacuolar transport in salt tolerance has been provided by *Arabiodopsis thaliana* plants overexpressing a vacuolar H^+-PPiase (Gaxiola *et al.*, 2001). Transgenic plants overexpressing *AVP1*, coding for the vacuolar H^+-pyrophosphatase, displayed enhanced salt tolerance that was correlated with the increased ion content of the plants. These results have suggested that the enhanced vacuolar H^+-pumping in the transgenic plants provided additional driving force for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter. The paramount role of sodium compartmentation in plant salt tolerance has been further demonstrated in

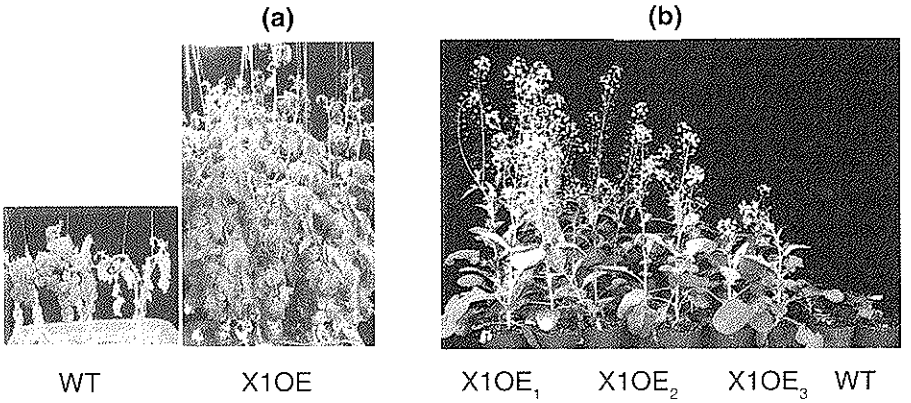

Figure 12.2. (a) Salt tolerance of wild-type (WT) tomato plants and transgenic plants overexpressing AtNHX1(X1OE) grown in the presence of 200 mM NaCl. Plants shown after 11 weeks of growth; (b) salt tolerance of wild-type (WT) and transgenic *Brassica* plants overexpressing AtNHX1 grown in the presence of 200 mM NaCl. Wild-type (WT) and homozygous plants showing high ($X1OE_1$), medium ($X1OE_2$), and low ($X1OE_3$) levels of expression were grown in the presence of 200 mM NaCl. Plants shown after 10 weeks of growth.

transgenic tomato plants overexpressing AtNHX1, the *Arabidopsis thaliana* vacuolar Na^+/H^+ antiporter (Zhang and Blumwald, 2001). The transgenic tomato plants grown in the presence of 200 mM NaCl were able to grow, flower, and set fruit (*Figure 12.2a*). Although the leaves accumulated high sodium concentrations, the tomato fruits displayed very low amounts of sodium (Zhang and Blumwald, 2001). Similar results were obtained with transgenic *Brassica napus* (canola) overexpressing *AtNHX1* (Zhang *et al.*, 2001) (*Figure 12.2b*). Leaves of transgenic plants grown in the presence of 200 mM NaCl accumulated sodium to up to 6% of their dry weight, but the seed yields and oil quality were not affected, demonstrating the potential of this technology for agricultural use in saline soils.

Synthesis of compatible solutes

The cellular response of salt-tolerant organisms to salinity stress (both long- and short-term) includes the synthesis and accumulation of a class of osmoprotective compounds known as compatible solutes. These relatively small, organic osmolytes include amino acids and derivatives, polyols and sugars, methylamines, etc. These osmolytes can stabilize proteins and cellular structures, and can increase the osmotic pressure of the cell (Yancey *et al.*, 1982; Yancey, 2001). This response is homeostatic for cell water status and protein integrity, which is perturbed in the face of soil solutions containing higher amounts of NaCl and the consequent loss of water from the cell. The accumulation of osmotically active compounds in the cytosol increases the osmotic potential to provide a balance between the apoplastic solution, which itself becomes more concentrated with Na^+ and Cl^- ions, and the vacuolar lumen, which in halophytes can accumulate up to 1 M Na^+ (and Cl^-). For a short-term stress, this may provide the cells with the ability to prevent water loss. However, for

continued growth under salinity stress, an osmotic gradient (towards the cytosol) must be kept in order to maintain turgor, water uptake, and facilitate cell expansion.

Because compatible solutes are non-toxic, the interchangeability of these compounds between species has held much interest (*Table 12.1*). The most recent examples include the engineering of ectoine synthesis (with enzymes from the halophylic bacterium *Halomonas elongata*) in plants (Ono *et al.*, 1999; Nakayama *et al.*, 2000), and trehalose synthesis (which occurs in bacteria, yeast, and in extremely desiccation-tolerant plants) (Goddijn and van Dun, 1999) which has been installed in potato (Yeo *et al.*, 2000). An intriguing report on the improved tolerance to salinity in tobacco expressing yeast invertase in the apoplast highlights the potential of manipulating sucrose metabolism (Fukushima *et al.*, 2001). Those authors reported improved salt tolerance of transgenic tobacco plants expressing a yeast invertase in their apoplastic space, and concluded that the changes in sucrose metabolism in the transgenic plants protected the photosynthetic apparatus under salt stress. The overexpression of polyols, such as mannitol (Tarczynski *et al.*, 1993) and D-ononitol (Sheveleva *et al.*, 1997) have been shown to contribute to enhanced drought and salt tolerance in transgenic tobacco plants.

Table 12.1. Increased stress tolerance in plants overexpressing genes encoding enzymes mediating osmolyte synthesis

Gene	Gene product	Source	Osmolyte	Target plant	Reference
ectA, ectB, ectC	L-2,4-diaminobutyric acid acetyltransferase, L-2,4-diaminobutyric acid transaminase, L-ectoine synthase	*Halomonas elongata*	Ectoyne	Tobacco cells	Nakayama *et al.*, 2000
otsA otsB	Trehalose-6-P synthase Trehalose-6-P phosphatase	*E. coli*	Trehalose	Tobacco	Pilon-Smits *et al.*, 1998
TPS1	Trehalose-6-phosphate synthase	*S. cerevisiae V. acontifolia*	Trehalose	Potato	Yeo *et al.*, 2000
P5CS	Δ^1-Pyrroline-5-carboxylase	*Arabidopsis thaliana*	Proline	Tobacco	Kishor *et al.*, 1995
ProDH	Proline dehydrogenase	*Arthrobacter globiformis*	Proline	*Arabidopsis*	Nanjo *et al.*, 1999
CodA	Choline oxidase	*Arthrobacter panescens*	Glycine betaine	*Arabidopsis*, rice, *Brassica juncea*	Hayashi *et al.*, 1997; Sakamoto *et al.*, 1998; Prasad *et al.*, 2000
COX	Choline oxidase	*E. coli*	Glycine betaine	*Arabidopsis*, tobacco and *Brassica napus*	Huang *et al.*, 2000
BetA, BetB	Choline dehydrogenase Betaine aldehyde dehydrogenase	*E. coli*	Glycine betaine	Tobacco	Holmstrom *et al.*, 2000
PEAMT	Phosphoethanolamine N-methyltransferase	Spinach	Glycine betaine	Tobacco	McNeil *et al.*, 2001
IMT1	Myo-inositol-*O*-methyl transferase	*M. chrystallinum*	D-ononitol	Tobacco	Sheveleva *et al.*, 1997
MtlD	Mannitol-1-phosphate dehydrogenase	*E. coli*	Mannitol	Tobacco	Tarczynski *et al.*, 1993

The enhancement of proline and glycine betaine synthesis in target plants has received more attention (Rontein *et al.*, 2002). Two themes have emerged from the results of these combined efforts: i) there are metabolic limitations on the absolute levels of the target osmolyte that can be accumulated; and ii) the degree to which the transformed plants are able to tolerate salinity stress is not necessarily correlative with the amounts of the osmoprotectants attained. The metabolic limitations on increasing the concentration of a given osmoprotectant is well illustrated with both proline and glycine betaine. Initial strategies aimed at engineering higher concentrations of proline began with the overexpression of genes encoding the biosynthetic enzymes P5C synthase (P5CS) and P5C reductase (P5CR) that catalyze the two steps between the substrate, glutamic acid, and the product, proline. P5CS overexpression in tobacco dramatically elevated free proline in transgenic tobacco (Kishor *et al.*, 1995). However, the regulation of free proline is not straightforward. Proline catabolism, via proline dehydrogenase (ProDH), is upregulated by free proline, and there is strong evidence for the inhibition of P5CS by free proline (Roosens *et al.*, 1999). Recently, a two-fold increase in free proline was achieved in tobacco plants transformed with a *P5CS* modified by site-directed mutagenesis (Hong *et al.*, 2000). This modification alleviated the feedback inhibition by proline on the P5CS activity and resulted in an improved germination and growth of seedlings under salt stress. Free cellular proline levels are also transcriptionally and translationally controlled. P5CR promoter analysis revealed that P5CR transcripts have reduced translational initiation. A 92 bp segment of the 5'UTR of P5CR was sufficient to provide increased mRNA stability and translational inhibition under salt stress of the GUS reporter that had been installed 3' to this small region (Hua *et al.*, 2001). These results highlight the complex regulation of P5CR during stress and the importance of stability and translation of mRNA during salt stress. An alternative approach to attain significant free proline levels, where antisense cDNA transformation was used to decrease ProDH expression, was utilized (Nanjo *et al.*, 1999). Levels of proline in the transgenic *Arabidopsis* were twice (100 μg/g fresh weight) that of control plants grown in the absence of stress, and three times higher (600 μg/g fresh weight) than in control plants grown under stress. The high levels of proline were correlated with an improvement in tolerance to salinity, albeit for a short duration exposure to 600 mM NaCl.

There has been considerably more experimentation directed at the engineering of glycine betaine synthesis than for any other compatible solute. Unlike proline, glycine betaine degradation is not as significant in plants (Nuccio *et al.*, 2000), but the problems of metabolic fluxes, compounded with the compartmentation of the substrate and product pools, has made the engineering of appreciable levels of glycine betaine problematic. In plants that are naturally accumulators (spinach and sugarbeet), glycine betaine synthesis occurs in the chloroplast, with two oxidation reactions from choline to glycine betaine. The first oxidation to betaine aldehyde is catalyzed by choline monooxygenase (CMO), an iron-sulphur enzyme. Betaine aldehyde oxidation to glycine betaine is catalyzed by betaine aldehyde dehydrogenase (BADH), a non-specific soluble aldehyde dehydrogenase (Rathinasabapathi, 2000). In *E.coli*, these reactions are cytosolic, but the first reaction is catalyzed by the protein encoded by the *betA* locus, choline dehydrogenase (CDH), which is an NAD^+-dependent enzyme, like BADH, which in *E.coli* is encoded by the *betB* locus. However, in some microorganisms, like *Arthrobacter globiformis*, the two oxidation steps are catalyzed

by one enzyme, choline oxidase (COD), which is encoded by the *codA* locus (Sakamoto and Murata, 2000). The *codA* gene of *A. globiformis* offers an attractive alternative to the engineering of glycine betaine synthesis using the plant enzymes, as it necessitates only a single gene transformation event. This strategy has been employed for engineering glycine betaine synthesis in *Arabidopsis* (Hayashi *et al.*, 1997). The 35S promoter driven construct for transformation included the transit peptide for the small subunit of Rubisco so that COD would be targeted to the chloroplast. Improved salinity tolerance was obtained in transgenic *Arabidopsis* that accumulated, as a result of the transformation, 1 μmol/g FW glycine betaine. The same construct was used for the transformation of *Brassica juncea* (Prasad *et al.*, 2000), and tolerance to salinity during germination and seedling establishment was improved markedly in the transgenic lines. COX from *Arthrobacter panescens*, which is homologous to the *A. globiformis* COD, was used to transform *Arabidopsis*, *Brassica napus*, and tobacco (Huang *et al.*, 2000). This set of experiments differs from those above in that the COX protein was directed to the cytoplasm and not to the chloroplast. Improvements in tolerance to salinity and drought and freezing were observed in some transgenics from all three species, but the tolerance was variable among all three species. The levels of glycine betaine in the transgenic plants were not significantly higher than those of wild-type plants, but increased significantly with the exogenous supply of choline to plants, suggesting that the supply of choline is a significant constraint on the synthesis of glycine betaine (Huang *et al.*, 2000).

There are two commonalities in the results from these groups. The first is that the concentrations of glycine betaine in the transgenic plants were much lower than the concentrations seen in natural accumulators. Despite the fact that these levels are not high enough to be osmotically significant, a moderate (and significant) increase in tolerance to salinity and other stresses was conferred. This raises the possibility that the protection offered by glycine betaine is not only osmotic, which is a point raised by all three groups; this explanation was also offered by Bohnert and Shen (1999). Compatible solutes, including mannitol, may also function as scavengers of oxygen radicals, which may be supported by the results of Alia *et al.* (1999), where the protection of PSII in plants expressing *codA* was observed. A second possibility, not necessarily exclusive of the first, is that the increased level of peroxide generated by the COD/COX oxidation of choline causes an upregulation of ascorbate peroxidase and catalase (Holmstrom *et al.*, 2000), which may also improve the tolerance to salinity stress (Rontein *et al.*, 2002).

The second commonality is that the level of glycine betaine production in the transgenics is limited by choline, despite little changes compared to untransformed plants in the free choline pool. Because betaine synthesis takes place in the chloroplast, the free choline pool may not reflect its availability to the chloroplast, which may be limited in this compartment by the activity and/or abundance of choline transporters (see discussion below). However, a dramatic increase in glycine betaine levels (to 580 μmol/g DW in *Arabidopsis*) has been shown in the transgenic plants when they were supplemented with choline in the growth medium (Huang *et al.*, 2000). This limitation was not explored in the transgenic tobacco expressing *E. coli* enzymes CDH and BADH in the cytoplasm (Holmstrom *et al.*, 2000). Although these transgenic plants demonstrated an improved tolerance to salinity, glycine betaine levels were on the order of those mentioned above. Sakamoto and Murata (2000) do assert that, despite

the similarities in tolerance exhibited by transgenic plants engineered to synthesize betaine in either the chloroplast or cytoplasm, the site of synthesis of betaine may play a role in the degree of tolerance shown. Indeed, if the betaine present in these plants is localized primarily in the chloroplast, it may be present at significant concentrations (50 mM) (Hayashi *et al.*, 1997). However, Sakamoto and Murata (2000) downplay the limitation of the metabolic pool of choline on the levels of glycine betaine obtained in the engineered plants. They suggest that the choline oxidizing activity may be the limiting factor, which seems to be supported by Huang *et al.* (2000), who see that the levels of glycine betaine correlate with the levels of COX activity measured in each plant. The increase in glycine betaine with exogenous choline (mentioned above) argues against this notion.

Stronger evidence for the limitations of choline metabolism has been presented (McNeil *et al.*, 2001). By overexpressing spinach phosphoethanolamine N-methyltransferase (PEAMT), which catalyzes the three methylation reactions required for the conversion of phosphoethanolamine to phosphocholine, up to a 50-fold increase in free choline has been obtained. This was shown to lead to an increase in glycine betaine levels (+60%) in plants that were already expressing spinach CMO and BADH in the chloroplast. However, the addition of ethanolamine to the plant growth medium further increased choline and glycine betaine levels, showing that the metabolic flux through this pathway is also limited by the supply of ethanolamine. That glycine betaine levels were not increased further may be due to the limitation of choline supply to the chloroplast. PEAMT is itself inhibited by phosphocholine, so further engineering efforts will include: the modification of PEAMT to remove this inhibition (McNeil *et al.*, 2001), increasing the supply of ethanolamine by overexpression of serine decarboxylase, and resolving the compartmentation problem of choline supply and choline oxidation, either by use of choline oxidation in the cytoplasm or by finding the appropriate transporters to improve choline supply to the chloroplast (Rontein *et al.*, 2002).

Antioxidant protection

A secondary aspect of salinity stress in plants is the stress-induced production of reactive oxygen species (ROS), including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). ROS are a product of altered chloroplast and mitochondria metabolism during stress. These ROS cause oxidative damage to different cellular components, including membrane lipids, protein, and nucleic acids (Halliwell and Gutteridge, 1986). The alleviation of this oxidative damage could provide enhanced plant resistance to salt stress. Plants use low molecular mass antioxidants such as ascorbic acid and reduced glutathione, and employ a diverse array of enzymes such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione S-transferases (GST), and glutathione peroxidases (GPX) to scavenge ROS.

Transgenic rice overexpressing a yeast mitochondrial Mn-dependent SOD has been shown to display enhanced salt tolerance (Tanaka *et al.*, 1999). The overexpression of a cell wall peroxidase in tobacco plants improved germination under osmotic stress (Amaya *et al.*, 1999). Transgenic tobacco plants overexpressing both GST and GPX displayed improved seed germination and seedling growth under

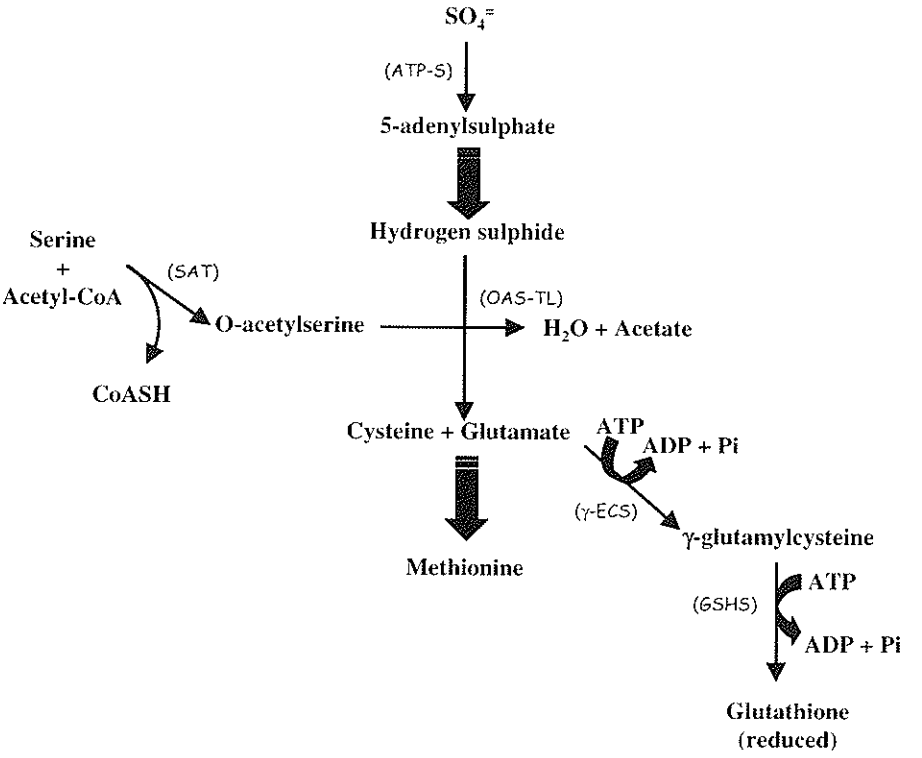

Figure 12.3. S-assimilation and the biosynthesis of cysteine and glutathione in plants. ATP-S = ATP-sulphurylase; SAT = serine acetyl transferase; OAS-TL = *O*-acetylserine(thiol)lyase; γ-ECS = γ-glutamyl-cysteine synthetase; GSHS = glutathione synthetase.

stress (Roxas *et al.*, 1997). Subsequent studies (Roxas *et al.*, 2000) demonstrated that in addition to increased GST/GPX activities, the transgenic seedlings contained higher levels of glutathione and ascorbate than wild-type seedlings, displayed higher levels of monodehydroascorbate reductase activity, and the glutathione pools were more oxidized. These results would indicate that the increased glutathione-dependent peroxidase scavenging activity and the associated changes in glutathione and ascorbate metabolism led to reduced oxidative damage in the transgenic plants and contributed to their increased salt tolerance.

During salt stress, plants display an increase in the generation of H_2O_2 and other ROS (Gueta-Dahan *et al.*, 1997; Roxas *et al.*, 2000). The major substrate for the reductive detoxification of H_2O_2 is ascorbate, which must be continuously regenerated from its oxidized form. A major function of glutathione in protection against oxidative stress is the reduction of ascorbate via the ascorbate–glutathione cycle, where GSH acts as a recycled intermediate in the reduction of H_2O_2 (Foyer and Halliwell, 1976). Recently, Ruiz and Blumwald (2002) investigated the enzymatic pathways leading to glutathione synthesis during the response to salt stress of wild-type and salt-tolerant *Brassica napus* L. (canola) plants overexpressing a vacuolar Na^+/H^+ antiporter (*Figure 12.3*).

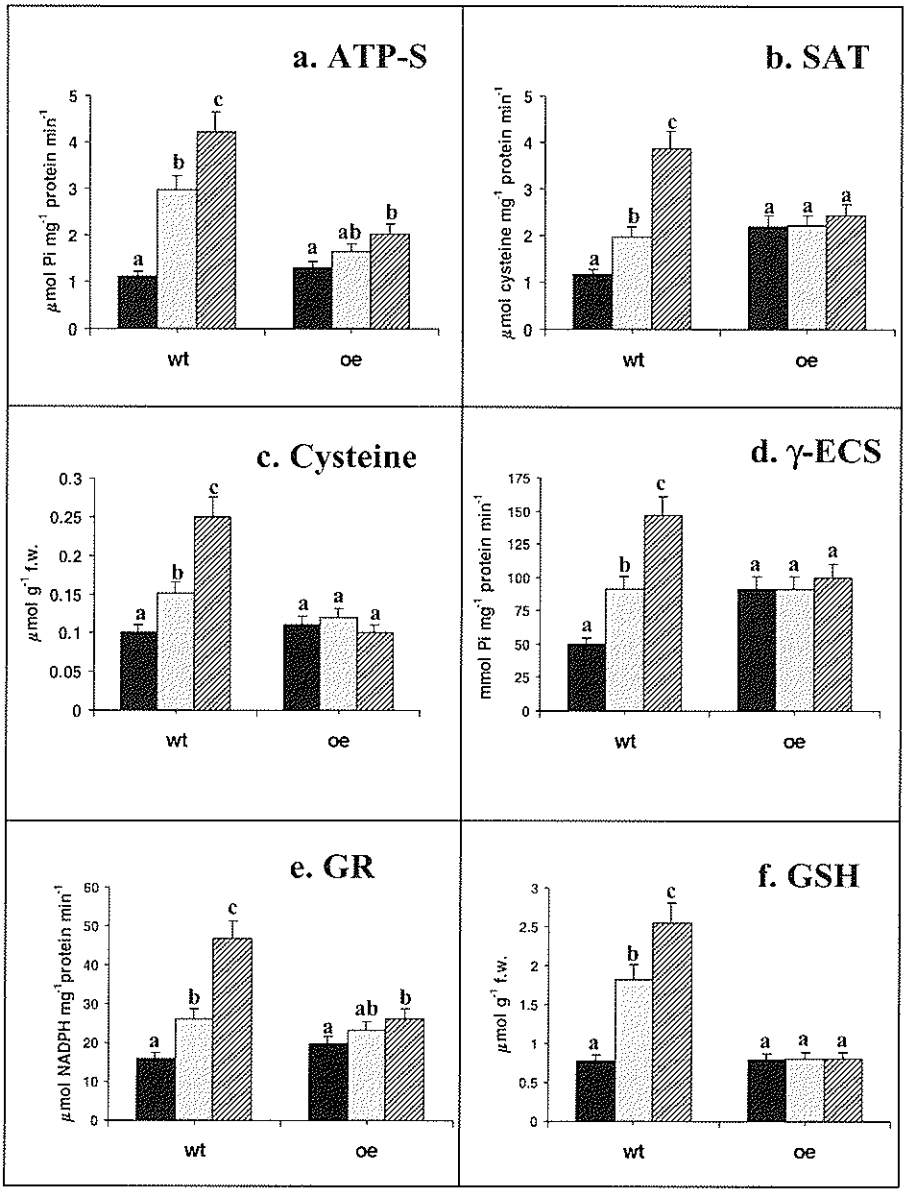

Figure 12.4. Assimilation of sulphur and biosynthesis of cysteine and glutathione in wild-type (wt) and transgenic canola (*Brassica napus*) (oe) plants exposed to 0 mM NaCl (black bars), 75 mM NaCl (grey bars) and 150 mM NaCl (hatched bars). Values are the mean ± SE ($n = 6$). Based on *t*-test analysis, values on bars headed by different letters differ significantly ($P < 0.05$).

In wild-type plants, the increase in NaCl in the growth medium led to a marked increase in key enzymes involved in cysteine synthesis (the crucial step for assimilation of reduced sulphur into organic compounds such as glutathione) (Noctor *et al.*, 1998) – ATP sulphurylase (ATP-S) and serine acetyl transferase (SAT) – and cysteine

content (*Figure 12.4*). In salt-tolerant plants, only minor changes in these activities were detected. Salt stress also induced a marked increased in γ–glutamyl-cysteine synthetase (γ-ECS) and gluthatione reductase (GR) in wild-type plants, with the concomitant increase in GSH content. These activities were unchanged in the transgenic salt-tolerant plants, and their GSH content did not change with salt stress (*Figure 12.4*). These results clearly showed that salt stress induced an increase in the assimilation of sulphur, and the biosynthesis of cysteine and GSH aimed to mitigate the salt-induced oxidative stress. The small changes seen in the transgenic plants overexpressing the vacuolar Na^+/H^+ (Zhang and Blumwald, 2001) suggest that the accumulation of excess Na^+ in the vacuoles (and the maintenance of a high cytosolic K^+/Na^+ ratio) greatly diminished the salt-induced oxidative stress, highlighting the important role of Na^+ homeostasis during salt stress.

Twenty years ago, Epstein (1983) argued for the development of salt-tolerant crops with truly halophytic responses to salinity, i.e. accumulation of salt, in which the consumable part is botanically a fruit, such as grain or berries or pomes. In these plants, Na^+ ions would accumulate mainly in their leaves, and since the water transport to the fruits and seeds is mainly symplastic, much of the salt from these organs would be screened. Clearly, the combination of the traits reviewed here is the next step towards the improvement of plant salt tolerance and the generation of salt-tolerant crops. Thus, engineering the accumulation of salt in vacuolated cells, together with the active extrusion of sodium ions from non-vacuolated cells (i.e. young and meristematic tissue), will allow the maintenance of a high cytosolic K^+/Na^+ ratio. In combination with enhancing the production of compatible solutes, this will generate transgenic crop plants able to tolerate and grow in high soil salt concentrations.

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